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REVIEW

Adjuvants: friends in vaccine formulations against infectious diseases

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ABSTRACT

Infectious diseases represent a major cause of deaths worldwide. No vaccine or effective treatment exists nowadays, especially against intracellular pathogens. The increase in multiple drug and superbug antibiotic resistance strains, excessive medication, or misuse of drugs has prompted the search for other safe and effective alternatives. Consistent with this, adjuvants (Latin word "adjuvare": "help or aid") co-administered (Exo) in vaccines have emerged as a promising alternative to initiate and boost an innate, downstream signal that led to adaptative immune response. Nowadays, a promising model of strong immunogens and adjuvants at mucosal sites are the microbial bacterial toxins. Other adjuvants that are also used and might successfully replace aluminum salts in combination with nanotechnology are CpG-ODN, poly IC, type I IFNs, mRNA platforms. Therefore, in the present review, we focused to revisit the old to the new adjuvants compounds, the properties that make them friends in vaccine formulations against infectious diseases.

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1. Introduction

Infectious diseases remain the most common cause of death in children less than 5 y of age[1]. Common infectious diseases such as diphtheria, tetanus, poliomyelitis, smallpox, and measles were able to prevent using relatively simple vaccines that stimulated robust antibody responses, except for malaria, tuberculosis, and human immunodeficiency virus (HIV) for whose there are not at present any vaccine or effective treatment and these pathogens are not effectively prevented by antibodies alone but long-lasting cellular response[1]. Furthermore, the emergence of new resistance mechanisms, the excessive and misuse of antibiotics in a clinic, the decrease in the development of antibiotics in the industry are urgent issues that represent challenges that lead us to consider adjuvants in vaccines candidates can enhance and boost innate and adaptative long memory response, precisely against intracellular pathogens.

The host–pathogen interaction could be visualized as a chemical reaction in which each element reacts by triggering molecular and genetic events that will transmit intracellular signals from the cell surface membrane to the nucleus, leading to changes in gene expression and inducing a robust effector and memory immune response, while also bringing about mechanisms that will change the course of the interaction toward a higher susceptibility or resistance (establishment of the infection) (Figure 1). On one side, host response overcomes the mechanisms of evasion developed by pathogens, through the induction of innate and adaptive immune responses (Figure 1) while on other hand, the pathogens adjust and fitness physiological and genomic program for survival and adaptation (Figure 1). What might be the potential role of the adjuvants compounds in the interaction host-pathogen? A key function of the adjuvants compounds is to initiate innate immune response (recognition receptor – ligand) that trigger the inflammatory response (cytokines) to the infection. In other words, adjuvants, enhance pathogen recognition and eliciting a response similar to the natural innate immune response. Thereby, adjuvants (from the Latin word "adjuvare" meaning "help or aid"), defined as compounds or molecules that can promote and enhance immune response – humoral or cellular,[2–5] either at the interface of the systemic or mucosal compartment.[2,6–9]

The repertoire of the adjuvants (endo-exo) in nature is broad from herbaceous secondary metabolites to antimicrobial peptides. The former is one of the effective mechanisms to kill bacteria – which could be induced upon natural infections (i.e. human defensins), but it could also be obtained from bacterial and marine sources.[10] In general, the world of adjuvant is not limited to aluminum salts (Alum salts mostly induce Th2 type immune responses and therefore, mostly enhance antibody responses) on the contrary, it has extended to other compounds derived from fruits, vegetables, and other types of plants like the *Quillaga saponaria*, QS-21 which a saponin of amphiphilic structure, formed by a structure of a carbón skeleton derived from squalene bonded to sugar residues (1, 2 o 3) with a relatively lipophilic a glycine moiety. The adjuvants compounds can be also of marine, or bacterial origin, such as recombinant subunits of bacterial toxins, such as heat-stable enterotoxins from *Vibrio cholerae*, or *Escherichia coli* (CTX/LTX), *Bacillus thuringiensis* Cry proteins, type I interferon (IFNs), Heparin-binding hemagglutinin adhesion (HBHA), Monophosphoryl Lipid A (MPLA), Unmethylated oligonucleotides (CpGODN) able to bias and balance the Th cellular immune response between the Th1 type and Th2 immune response. Poly(I: C) (Polyinosic: polycytidylic acid), compounds that can participate in dendritic cell maturation.

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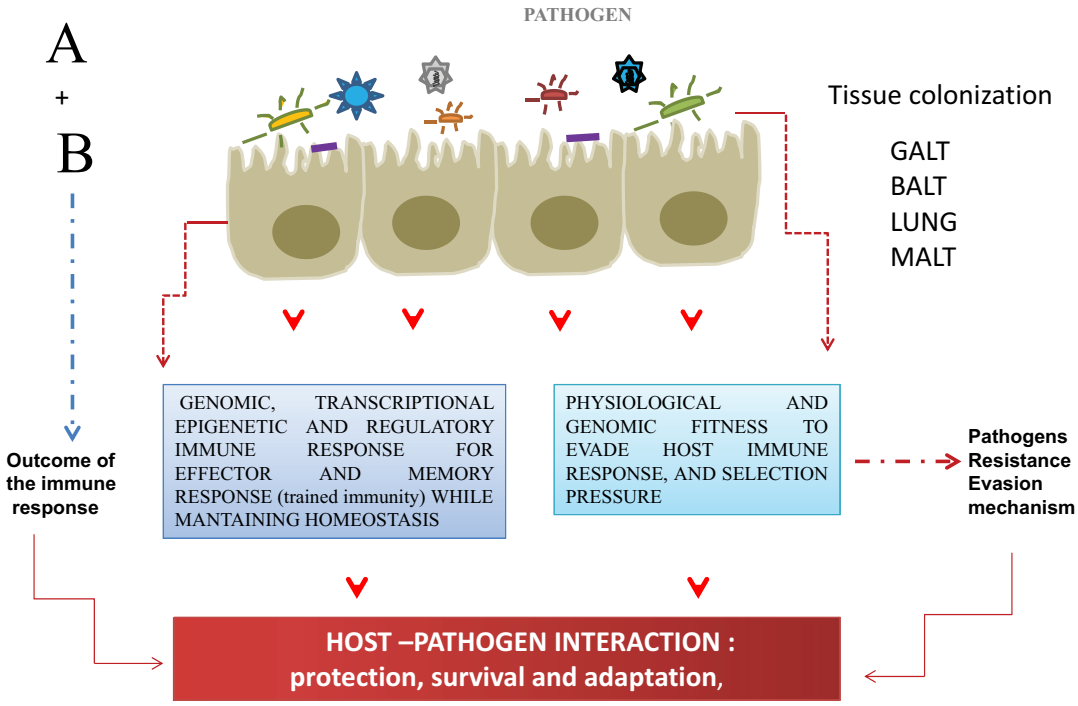

Figure 1. Most of the threatening infectious disease is targeted at the mucosal sites (around 400 mt2), therefore, the interaction at these sites constitutes the first line of the host defense. Upon host–pathogen interaction, a differential outcome for each other is the results of A + B, the product of this interaction, can be visualized as the outcome of the transcriptional and genomic program for the induction of protective adaptive immune response (HOST).; and for another side as an adjustment and fitness of the genomic program to develop evasion mechanism (PATHOGEN) for the successful establishment, survival, and adaptation into the host.

(Figure 2). In addition, calcium phosphate, chitosan have been replaced with aluminum salts because they bias Th cellular immune response toward Th1. Furthermore, the use of anti-inflammatory adjuvants in vaccine formulation to modulate the host response to pathogens toward T helper type (Th) Th2 or Th1 response, and the balance Th1/Th2 will dictate the outcome of humoral and effector cellular immune response. Thus, adjuvants in vaccine formulation as immune potentiators considering populations that are poor responders, elderly or vulnerable can influence positively in the induction of neutralizing antibody response and of CD8 + T lymphocytes (producers of granzymes, perforins) that are enough to limit virus or bacterial replication and establishment in the host cells.[2,6–8,11–15] Therefore, adjuvants are essential components of most clinically used vaccines. This is because the majority of non-living vaccines are relatively poor inducers of adaptive immunity unless effective adjuvants are co-administered. It is true not all vaccines need adjuvants, vaccines containing whole pathogens (live attenuated or inactivated), contain a heterogeneous mixture of diverse antigens and other pathogen components that act as intrinsic adjuvants, thereby these vaccines are capable of initiating innate immunity, which drives subsequent adaptive responses that lead to successful clearance of the pathogen. A problem is these vaccines are not suitable when natural infection itself does not confer long-standing immunity or when the pathogen is unable to be grown in culture. Thus, modern vaccines containing a limited number of purified antigens, which are also often less immunogenic due to the removal of pathogen features of the organism, and therefore, it is necessary to use the adjuvants because these compounds can improve immune responses in populations where responses to traditional vaccines are typically reduced such as infants, elderly, vulnerable and immunocompromised individuals, Despite some potential risks and safety considerations with the use of adjuvants, it is thought that the selection of the type the dose, and the route of administration, these compounds are key elements of current vaccines that are in development. In the present review, we aimed to revisit the old to the new adjuvant compounds, the properties that make them a friendly and promising strategy in vaccine formulations against infectious diseases (intracellular pathogens).

2. Types and classification of adjuvants compounds

Adjuvants can be categorized into two types: 1. Compounds/molecules that directly stimulate the host immune system called "Immunostimulants" and 2. Compounds that act indirectly included in vaccine formulations with live-attenuated pathogens (bacteria, viruses, fungi); purified antigens (recombinant proteins); subunits (toxoid, split viruses, fragments of pathogens)[16,17] and schematized system that comprised of adjuvant plus vaccine or purified antigens plus adjuvant simple or in combination. Another adjuvant classification that has been well accepted[16–18] as adjuvants consists of four groups: 1) Release system (e.g. mineral salts, aluminum salts, calcium phosphate).[19–21] 2) Immunomodulators or immunopotentiators (e.g. MPLA, LPS (lipopolysaccharide), Flagellin, CpG, Poly IC, QS21; ISCOMs).[22–26] 3). Mucosal adjuvants (bacterial toxins.[9,15] 4). Adjuvants system, such as (AS01, AS03, AS04[9,22,25]; Figure 2). In more recent years, type I IFNs,[26–32] antimicrobial peptides;[33–42] chemokines;[43] and the use of

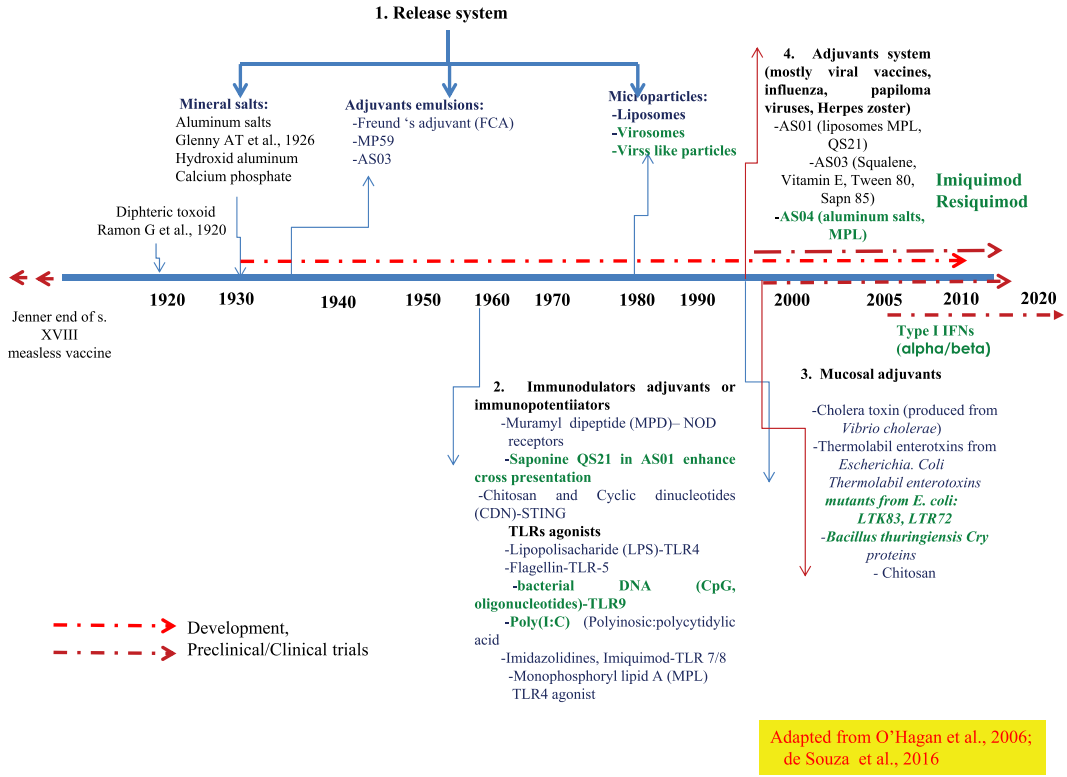

Figure 2. The repertoire of adjuvants compounds, categories, and development since 1920. A released system developed to target the vaccine subunits to the sites of the induction of the innate immune response (AS03, AS04, liposomes virosomes, viral-like particles). TLR agonist, immunostimulants (CpGODN, ISCOMS; Poly (I: C), MPL Flagellin; QS21, Imiquimod, Resiquimod). Of relevance is the availability and the potential of molecular mucosal adjuvants, -recombinant bacterial toxin, type I IFNs-that deserve further exploration and deep evaluation in phase I through phase III against intracellular pathogens.

nanoparticle[19–21] represent the new era of mucosal adjuvants[2,15–18] (Figure 2).

2.1. Immunological properties of the adjuvants

2.1.1. Release systems

Aluminum salts (alum) have been used as adjuvants with great success for almost a century and have been particularly effective at promoting protective humoral immunity. Aluminum salt/gel-based (alum) adjuvants remain the only standard versatile adjuvant licensed for human use in the United States. Alum can not induce a T helper type I (Th1) cell-mediated immune response that is important in fighting against certain viruses, bacteria, and parasites. Until now is the only approved adjuvants for human use, a Th2 type adjuvant that stimulates poor immunity to the elderly.[43] To note is that Aluminum-adjuvanted vaccines have not been successful in preventing infection due to intracellular pathogens. Another early adjuvant attempt was a mineral oil-in-water emulsion (Freund's incomplete adjuvant) which was considered too reactogenic for continued use in humans Adjuvants have been used for more than 90 y and are currently of more than 30 licensed vaccines from different manufacturers.[17,44] Alum exerts its immune-stimulatory activity by triggering the release of uric acid, a danger signal that amplifies the activation of DCs via the NALP3 inflammasome as shown by the increase in the co-stimulatory ligand CD86. This amplified DC activation leads to an immediate inflammatory response at the administration site, the generation of an adaptive cellular immune response, and a persistent Th2 immunity.[45,46] Alum adjuvants induce the release of interleukin1-beta (IL-1β) from macrophages and dendritic cells and that this is abrogated in cells lacking various NALP3 inflammasome components.[47]

The NALP3 inflammasome is also required *in vivo* for the innate immune responses to OVA in alum. The activation of the cellular immunity to OVA alum is initiated by monocytic dendritic cell precursors that induce the expansion of Ag-specific T cells in a NALP3 dependent way. It has been proposed that in addition to TLR stimulators, agonists of the NALP3 inflammasome, should also be considered as vaccine adjuvants[48–54] (Figure 3(a)).

In a recent study, it was described that alum also induces high-level production of uric acid *in vivo* and this increased level of uric acid was required for infiltration of inflammatory cells. Although how this is done and which cells generate or release uric acid upon alum administration are open questions, it suggests that the increased level of uric acid leads to an amplification of the NALP3 inflammasome activation and, thus, IL1-β secretion. Interestingly, uric acid was identified not only as one of the most potent danger signals released from dying cells but also as an excellent adjuvant.[53–55]

2.1.2. Immuno-stimulatory or immune-potentiators molecules

Adjuvants compounds that can enhance innate immunity. Adjuvants containing pathogen-associated molecular patterns act as ligands for TLRs. Thus, TLR9 was shown to be essential

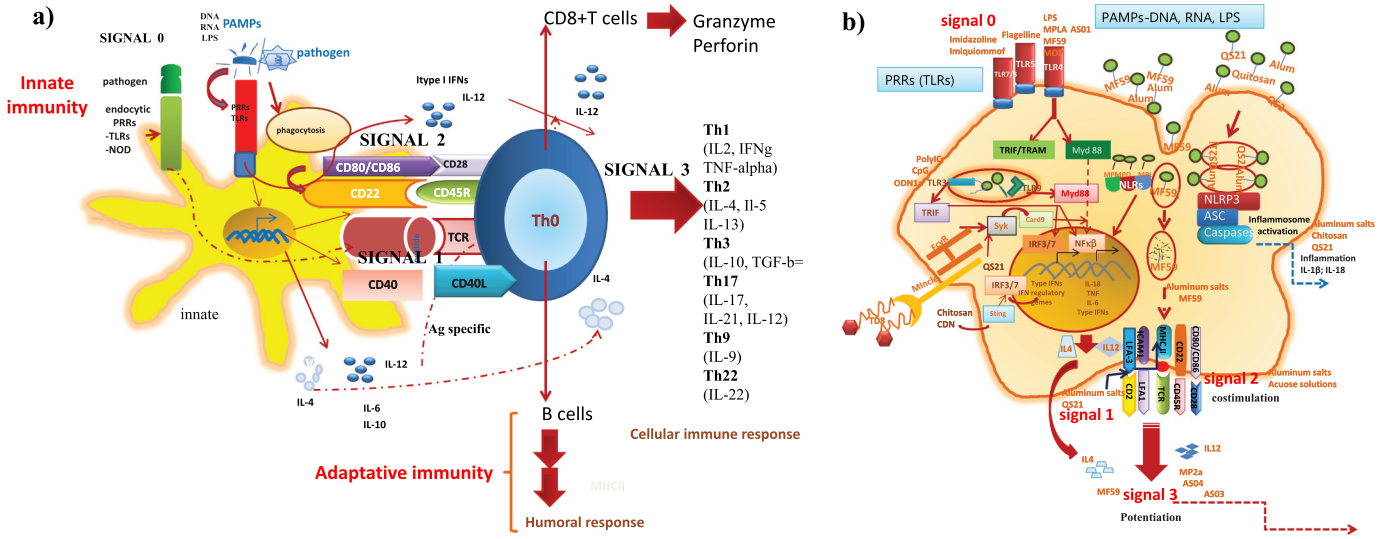

Figure 3. How adjuvant compounds in vaccine formulations initiate, boost and trigger the innate and adaptative immune response. The induction of the immune response occurs through the interaction of the mimic pathogen-associated molecular patterns (PAMPs) and the PRRS (e.g. TLRs, NLRs receptors) on antigen-presenting cells (APCs) (dendritic cells) that trigger an innate immune response leading to activation and maturation of APCs and initiation of downstream MyD88 signalization transduced to the nucleus, leading to pro-inflammatory response necessary to mount a specific durable and effective immune response by T CD4+ and T CD8 + lymphocytes (a). The mechanism of action of the adjuvants comprises a program of four signals which ended with the induction of the effector and memory cellular immune responses and thereby specific T and B cell responses. From the repertoire of adjuvants (TLR agonists, CpG-ODN, Poly(I: C, bacterial toxins), vehicles/carriers (ISCOMS, virosomes, liposomes), adjuvant system (AS03, AS04) as components of modern vaccines, which usually lack some of the components of the whole live microorganism, initiate the innate immune response by acting as PAMPs and thereby, participate by enhancing the interaction between the vaccine ((Ag) and the antigen-presenting cells (macrophages, dendritic cells, epithelial cells), by enhancing uptake, presentation, costimulation and activation, n of quality and magnitude of the activation of CD4 + T cells and CD8 + T cells and B cell differentiation to plasmacytoid B cells antibody producers, cytokines (TGF-β) and T cell homing molecules at MAL (mucosal-associated tissues) (b).

for the adjuvant effect of CpG oligonucleotides.[54] Alum adjuvants in contrast to bacteria-derived adjuvants do not activate TLRs. Alum adjuvants trigger activation of the NALP3 inflammasome. The potential of alum to trigger the NALP3 inflammasome leads to early activation of the innate cytokine IL-1β and an innate cellular immune response at the site of injection. Activation of the NALP3 inflammasome and the subsequent release of IL-1b leads to the recruitment of immature monocytes and DCs[47,56] (Figure 3A). Production of IL-1β also leads to the activation of inflammatory monocytes and their migration to the lymph nodes draining the peritoneum. Interestingly, MPL® is the first non-alum vaccine adjuvant obtained from a *Salmonella enterica* endotoxin, which accounts for significant widespread and clinical market acceptance. The stimulatory dose–response curves revealed that most preparations of MPL are much more active in mice than in human cell systems, this is because in human cells correlated with human TLR4 inhibitory activity that resulted in a partial agonist profile.[57] While the biodegradable adjuvant, MCT®, was developed for application in the niche area of allergy immunotherapy (AIT), also in combination with a TLR-4 adjuvant-MPL®-producing the first adjuvant system approach for AIT in the clinic.[58]

The Adjuvant System AS01 (a liposome-based vaccine adjuvant system containing two immunostimulants: 3-O-desacyl-4′-monophosphoryl lipid A (MPL) and the saponin QS-21. AS01 is efficient at promoting CD4 + T cell-mediated immune responses and is an appropriate candidate adjuvant for inclusion in vaccines targeting viruses or intracellular pathogens. AS01 has been selected for the clinical development of several candidate vaccines including the RTS, malaria vaccine, and the subunit glycoprotein E varicella-zoster vaccine (both currently in phase III. AS01 to improve adaptive immune responses. Enhancement of the adaptive immunity by AS01 depends on activated dendritic cells and that depends on synergistic activities of QS-21 and MPL.[59,60] MPL and aluminum salts are present in AS04, and both MPL and QS-21 are present in AS01 and AS02, which are liposome- and emulsion-based formulations, respectively. The licensing of two AS04-adjuvanted vaccines and the initiation of Phase III trials with an AS01-adjuvanted vaccine demonstrate the potential to develop new or improved human vaccines that contain MPL or MPL and QS-21.[61] In viral settings, it was compared AS01 versus other Adjuvant Systems in a candidate herpes zoster glycoprotein E subunit vaccine. It was evaluated formulated with AS01B, AS01E (50% less MPL and QS-21 than AS01B), AS03, or AS04 in C57BL6 mice primed with live-attenuated VZV. Four-weeks post-vaccination, the IgE-specific CD4 + T-cell response to gE/AS01B was 5.4, 2.8 and 2.2-fold greater than those to gE/AS03, gE/AS04 and gE/AS03, respectively ($p < .001$). Therefore in the VZV-primed mouse model, CD4 + T-cell responses to IgE were most enhanced by AS01.[62] In other vaccine formulations, liposomes containing monophosphoryl lipid A and QS-21 serve as an effective adjuvant for soluble circumsporozoite protein malaria vaccine FMP013 in a mouse model, C57BL6. FMP013 antigen in C57BL/6 mice formulated with two novel adjuvants of the Army Liposome Formulation (ALF) series and a commercially available adjuvant Montanide ISA 720 (Montanide) as a control. ALF is a liposomal adjuvant containing a synthetic monophosphoryl lipid A (3D-PHAD®). FMP013 was adjuvanted with ALF alone, ALF containing aluminum hydroxide (ALFA), or ALF containing QS-21 (ALFQ). Adjuvants ALF and ALFA induced similar antibody titers and protection against transgenic parasite challenges that

were comparable to Montanide. FMP013+ ALFQ also augmented the numbers of splenic germinal center-derived activated B-cells and antibody-secreting cells compared to Montanide. Further, FMP013+ ALFQ induced antigen-specific IFN-γ ELISPOT activity, CD4 + T-cells, and a TH1-biased cytokine profile.[63] Moreover, immunization with Virus-Like Particles Encapsulated in Monophosphoryl Lipid A and Liposomes there is a promotion of Cellular and Humoral Immunity against Foot-and-Mouth Disease Virus.[64] In the study, it was described that MPL/DDA-VLPFMDV could induce strong cell-mediated immune responses by inducing not only VLP-specific IFN-γ+ CD4+ (Th1), IL-17A +CD4+ (Th17), and IFN-γ+ CD8+ (activated CD8 response) T cells, but also the development of VLP-specific multifunctional CD4+ and CD8+ memory T cells co-expressing IFN-γ, TNF-α, and IL-2 (Figure 3(a,b)). In addition, MPL/DDA-VLPFMDV vaccine markedly induced VLP-specific antibody titers; in particular, induce greater Th1-predominant IgG responses than VLPFMDV only and DDA-VLPFMDV[65]. Furthermore, bacterial lipopolysaccharide (LPS) is toxic, and it has an excellent ability to mobilize innate immunity by Toll-like and other receptors and promote the maturation of dendritic cells.[66] However, its modified version of MPL is less toxic but a better adjuvant.[67,68] Finally, a liposome-based delivery system incorporating CpG (is the most promising TLR Ligand that stimulates TLR9 in a pathway requiring the adaptor MyD88, leading to the activation of dendritic cells (DCs)[2,23] which induces the rapid recruitment of neutrophils, enhances dendritic cell-associated Ag transport and influences the maturation of innate cells entering the afferent lymph, translated into an extended period of lymph node shutdown, the induction of IFN-γ-positive T cells, and enhanced production of Ag-specific Abs in a large animal model after vaccination of a dose comparable to that administered to humans.[65]

2.1.3. Mucosal adjuvants

2.1.3.1. Bacterial toxins. Bacterial toxins are protein antigens, a condition that will result with time in the production of neutralizing antibodies that would abrogate their adjuvanticity and a loss of vaccine efficacy. This observation applies also to carriers containing proteins like keyhole lymphocyanin (KLH) and viral particles, where antibodies against the carrier's protein-(s) may inhibit immune response signal against the conjugated immunogens by a process known as carrier induced epitopes suppression (CIES).[69] A group of bacterial toxins, e.g. *Vibrio cholerae*, cholera toxin (CTx), and *Escherichia coli* heat-labile enterotoxins (LTx). ADP-ribosylating enterotoxins as vaccine adjuvants[70–77] that are been considered for Alzheimer's disease (AD) development. Since most of the pathogens enter the body by mucosal sites, therefore, it is key to develop mucosal vaccines that prevent local infection or invasion of pathogens, able to induce or to mount significant innate and adaptive immune responses in terms of sIgA antibodies, a subclass of IgG and tissue-resident memory CD4+ CD8 + T cells, Adenosine diphosphate (ADP)-ribosylating bacterial enterotoxins, such as cholera toxin (CT) and *Escherichia coli* heat-labile toxins (LTs) remain as the most strong mucosal immunogen and adjuvants. Cholera toxin (Ctx) and its close relative, *Escherichia coli* heat-labile enterotoxin (ETx) have long been established as potent mucosal and systemic adjuvants. Nontoxic-B-subunit of ETx (ETxB) is a highly potent mucosal adjuvant capable of potentiating protective immunity to viral infection by triggering specific signaling processes in lymphocyte populations, modulate differentially their activation differentiation and survival.[9,70–77] Research on these toxins has been focused on their effects as mucosal adjuvants inducing Th2 type cellular immune response, their induced immunity depends on several factors, e.g administration route, and age of the animals. The wild types of toxins are toxic to human beings, however, the mutants or derivatives. The mechanism of LTB adjuvanticity of LTB was to enhance the turnover of dendritic cells (DCs) in the spleen and increase DC capacity to perform as antigen presentation cells (APCs) encountered with T cells. LTB also induces B and T cell clustering and delay/arrest in T–cell division following endocytosis or B cell receptor (BCR) uptake of antigen in a ganglioside (GM1)-mediated manner. A nontoxic mutant of CT that in young mice induced Th2 immunity, in aged mice induced both Th1 and Th2[74] Also it has been shown that other CT mutants induce Th17 type, a strong inflammatory response that may be damaging in AD vaccines. The enterotoxin LT is also being evaluated as an adjuvant for AD vaccines and because of the wild-type toxin's toxicity, several nontoxic mutants have been developed. Like with CT mutants, the type of immunity induced by LT, either Th2 or Th1/Th2 type, would depend on the routes used for immunization.[75] Therefore, LT mutants depending on various factors may induce Th1 or Th17 inflammatory immunity not convenient for artherosclerosis (AS) vaccine but against intracellular pathogens.[76] Recent advances[77] has pointed out that in the mechanism of adjuvanticity of thermolabile enterotoxin subunit B (LTB) is the immunogenicity and not the binding or the ADP-ribosylation activity that accounts for the observed adjuvanticity. *Escherichia coli* heat-labile enterotoxins B subunit is a more potent mucosal adjuvant than its closely related holotoxin, the B subunit of cholera toxin.[77] In a study, purified ETxB and CtxB were tested with hen egg lysozyme, and it was found that ETxB induced higher responses than CTxB, assessed by the induction of secretory antibody titers as well as by the stimulation of lymphocyte proliferation in the spleen and in draining lymph nodes, implying that both subunits should be considered independent in prospective vaccines[78] (Figure 2). Another toxin that has shown that act as adjuvants are the Cry proteins, obtained from *Bacillus thuringiensis (Bt)*, a soil bacteria that have been used for several decades as bioinsecticides.[65,66] However, in recent years, several pieces of evidence have indicated that they can induce adjuvant protective effects toward several parasites such as *Naegleria fowleri*,[78] metacestodes in ciscticercosis,[79] Brucella abortus[80], *Plasmodium falciparum*,[81] or enhance cellular immunity to *Mycobacterium Bovis Bacillus Calmette Güerin (BCG)*.[14] The mechanism of action remains to be elucidated and defined. Despite this, it is tempting to propose this bacterial toxin as a safe alternative to induce robustness humoral and cellular immune responses at the systemic and mucosal levels.

3. How adjuvants initiate and boost innate and adaptive immune responses as components of vaccine formulations

The adjuvants, as immune potentiators can initiate and boost innate immune response[16–18] through mimicking pathogen-

associated molecular patterns (PAMPs), that interact with the pattern recognition receptors (PRRS) (e.g. TLRs, NLRs) on antigen-presenting cells (APCs) (dendritic cells, macrophages, epithelial cells (Figure 3(a)), resembles a reaction receptor-ligand, that trigger an innate immune response leading to activation and maturation of APCs (e.g. dendritic cells) and initiation of downstream MyD88 signalization transduced to the nucleus, and leading to a pro-inflammatory response necessary to mount a specific durable, and long-term effective immune response upon the host–pathogen interaction (Figure 1) that will influence and impact onB and T lymphocyte population (CD4+ and CD8+) (Figure 3(a) and the Th subsets (Th1, Th2) (Figure 3(a)). – **How adjuvants compounds accomplished this task?** The mechanism of action of adjuvants is wide and diverse.[2,5–8,24,25,27] But in general, it is well accepted that could be accomplished through a four-signal mechanism of action[17–19](Figure 3(b)). The adjuvant molecule interacts with the receptors on innate immune cells, the antigen-presenting cells (macrophages, dendritic cells, epithelial cells, neutrophils)[6,9,11,12,15] or **Signal 0: a first encounter** that involves the initial interaction between the pattern of recognition receptors (PRRs) such as toll receptors (TLRs), other non-TLRs receptors; NOD-like receptors (NLRs); RIG-I-like-receptor (RLRS); dectin receptors or mannose lectin-like receptors[11,82–91] on the skin or mucosal innate cells (macrophages, dendritic cells, B cells, epithelial cells) and pattern associated molecular pathogens (PAMPs) (DNA, RNA, proteins, LPS) and/or the pathogen (Figure 3(a) and Table 1).

Signal 1: antigen (Ag) presentation (enhanced by aluminum salts, oils, emulsions) or antigen uptake and processing (MF59). The PRRS-Ag could be endocytosed or the pathogen itself could be endocytosed. All these processes would lead to the activation and maturation of the innate response and the activation of the key signalization pathways (Myd88, TRIF/TRAF, STING), from the cytosol to the nucleus (translocation of NF-kB), expression of IRF3/[7,12,29,30] and induce an inflammatory response mediated by cytokines (IL-6, IL4, IL12), and chemokines as well (Figure 3(b)).

Signal 2: costimulatory signal (enhanced by QS21, hydroxide aluminum, oil emulsions) or augmenting co-stimulatory molecules (CD80/CD86; CD40/CD40L) that make robust the antigen presentation to naive lymphocytes (CD4 + T cells[2,21–23]; Figure 3(b)).

Signal 3: the signal of potentiation for the differentiation of naive CD4 + T cells, toward Th1/Th2 (helper 1 T cells/helper 2 T cells) [MP2a, ASO4 potentiates for Th1-type cellular immune responses while MF-59, and hydroxide aluminum for Th2-type cellular immune response (Figure 3(b)] and the different Th subsets [(Th17, Th9, Th22, Thf, follicular helper T cells, Tregs, regulatory T cells)] cytokine producers of IL-12, IL-17, IL-10, IL-22, IFN-gamma (IFN-γ) product will actívate macrophages (MØs), NK, NKT cells, CD8 + T cells (Cytotoxic T Lymphocyte); activation and differentiation of B cell to plasmatic B cells, antibody producers (neutralized or opsonized Abs)[2–8,11,15,16] (Figure 3(b)). Moreover, Tregs which express CTLA4[67,88,89] and FoxP3 produce IL-10 and TFG-beta (TGF-β), leading to a highly regulated immune response. In addition, direct antigen presentation of intracellular bacteria, viruses, protozoans o MHC-I-pathway to CD8 + T cell activation, differentiation to CD8 + T cell effectors (expression of FASL and perforin and granzyme production), which enabled the killing of

Table 1. Approved TLR agonist as adjuvants.

PRRs Receptor		PAMPs Natural Ligand	(Adjuvant) PAMPs	
TLRs	TLR1/2	pppRNA dsRNA	Triacyl lipopeptides	
	TLR2/6		Synthetic Pam3Cys Diacyl lipopeptides	MPLA(C)
	TLR2		Pam2Cys	AS01 (C)
	TLR3		Pam3Cys	AS02 (C)
	TLR4		Poly(I:C)	AS04 (C)
	TLR5	LPS	LPS, AS04 (MPL)	RC-528 (C)
	TLR7		Flagellin	PolyI:C and derivatives(T)
	TLR8		Imiquimod	
	TLR9		Resiquimod	
NLRs	NOD1/NLRC1	Flagellin	Resiquimod	Flagellin (T)
	NOD2/NLRC2		CpG-ODN	
	NLRP1	dsRNA	DAP	CpG-ODN(T)
	NLRP3		MDP	Polypropylene sulfide)NP surface
	IPAF/NLRC4	ssRNA	Toxoid, MDP	Conjugated to pGODN
	NAIP5		Alum, MDP, ATP	Prophylatic and therapeutic HDM
			Flagellin	Model (mouse/*in vivo*) (versus soluble
			Flagellin	CpG-ODN)
RLRs	RIG-1	CpGDNA	DNA vectors	-QbG10(VLPs encapsulating A-type
	MDA5		Poly(I:C)	CpG-ODN)
CLRs	Dectin-1		Flagellin. β -	Human clinical trial (versus placebo)
	Mincle		glucan/zymosan	
			CAF01	

Luchner et al., 2021

infected macrophages (Figure 3(b)). A similar action of the mucosal adjuvants, as well as those adjuvants of natural origin, marine or plant-derived (e.g. *Quillaga saponaria*) (Figure 2) can augment the immune responses to soluble antigens to contra rest the host's self-tolerance.[12,22,86] It has been suggested that this type of adjuvants boosts the protective immunity and promotes specific humoral and cellular immune responses by following the three signal mechanism actions: signal 0, 1, and 2.[89] Thus, when they are co-administered with novel vaccines, such as those based on attenuated microorganism or recombinant bacteria, virus, or vaccines in proper vehicle (liposomes, virosomes, ISCOMS) formulations, like oil emulsions (Figure 3(b)); adjuvants can exert a positive influence on antigen presentation, differentiation of naive CD4 + T cells, memory, and effector cellular immune responses[2,7,9,30,43](Figure 3 (b)). Moreover, adjuvants based on AS03, AS04, MF59 could enhance **signal 0** and **signal 1** (antigen uptake, processing), whilst bacterial toxins (e.g. cholera toxin of *Vibrio cholera*); thermolabile enterotoxins of *Escherichia coli*-their B subunits;[70–77] *Bt* Cry proteins[14] and Quitosan[11,16,85] have a role in adaptive antigen-specific immune response (**signal 2**: antigen presentation, co-stimulatory molecules). Th1 or Th2 CD4 + T cells within the secondary lymphoid tissues follicle elicited cytokines, other than TGF-β during the inflammatory or pathogen-induced reactions produced by Th1 or Th2 CD4 + T cells, which may ensure that näive B cells are committed toward IgG2 (IFN-γ) or IgE (IL-4), maturing into gut-homing IgG2 or IgE producing plasma cells. IL-4 and TGF-β induce surface IgM-positive (sIgM+) B cells which switch to IgE and IgA. TGF-β1 could induce sIgM to sIgA B-cell. In humans, anti-CD40 stimulation of tonsillar B cells, together with TGF-β1 in the presence of IL-10, stimulates IgA synthesis. Differentiation of sIgA+ B cells into IgA-producing plasma cells is dependent on IL-5 and IL-6.[2,37] (Figure 3(a,b)). In addition, the expression of T cell homing molecules plays a major role in the common mucosal immune system, which enables secretory IgA antibodies (SIgA) to be present in distal sites as in upper airways[5,6,11,12,82–86] (Figure 3(a,b)), and thereby be present in the frontline defense at mucosal sites, this represents a friendly action adjuvants compounds (Figure 1).

4. Adjuvants in vaccine formulations

Adjuvants compounds as components of vaccine candidates can act or mimic pathogen-associated molecular patterns (PAMPs) in the vaccine formulation, such that vaccine components are identified as a threat and so, trigger an innate immune response through a variety of mechanisms with

a) activation and maturation of APCs and initiation of downstream adaptive immun e response (Table 1).[43]

b) Adjuvants can increase the magnitude and durability of the response achievable using purified subunit antigen.

c) Adjuvants can reduce the number of antigens contained in individual vaccine doses. PAMPs that are typically associated with infections and facilitate target vaccines to their local action at the systemic and mucosal sites, and mostly at mucosal compartments since most of the pathogens enter via the mucosal routes.

The actual inclusion of adjuvants compounds in modern vaccine development is evident (Figure 2 and Tables 2 and 3) since there are two challenges that the science of vaccination has to deal with it: One is one related to the characteristics of the pathogen itself, and the other related to the characteristics of the population (infants, young, elderly, vulnerable, immunocompromised individuals).[16–18,43] First, related to the pathogen, among them, the evasion mechanism of the intracellular pathogens, the antigenic drift, the multiple serotypes, latent infection disease, and/or the short period of protection. The second one, related to the population features, like elderly adults with immunosenescence, populations with chronic disease, those immunosuppressed, and the infants with an immature immune system.[16,17,43] The adjuvants in vaccine formulations should also overcome several other important issues. The research and development, preclinical studies (animal studies) are very worthy to start the assessment of the safety of the adjuvant plus vaccine, to follow with phase I, II, III studies all of which constitute the pre-license stage before passing to the post-licensed stage that includes among others studies, phase IV safety studies, epidemiological studies. The benefit-risk profile should also be under constant reevaluation to warrant the safety and the feasibility of the final product. The potential safety concerns that have been described around the development of adjuvanted vaccines are the reactogenicity (swelling, pain, redness, general symptoms like fever, fatigue), especially it has been observed at the site of injection, the symptoms are from mild-to-moderate, despite this, all licensed adjuvanted vaccines have shown more a favorable benefit:risk ratio than adverse.[15–17,43] This is the reason by which all the time this ratio has reevaluated to overcome any problem, especially in the elderly people. Another safety concern is the immune-mediated disease, due to exposure to vaccination in susceptible individuals, because of the adjuvant immune-stimulants properties that can lead to unwanted immune responses. The World Health Organization (WHO) encouraged animal and epidemiological studies, and prolonged follow-up studies after vaccination to evaluate adverse effects. Increasing efforts have been made to identify the risk of immune-mediated disease after vaccination with adjuvanted vaccines. Other key issues that are around the public confidence in vaccine safety profiles and efficacy are the trust in companies and agencies that manufacture it, and the science underlying vaccine research and development, because how to work the vaccines not always is known. To understand how the adjuvants can augment the innate immune response and therefore the adaptive immune response will assure and enable the development of new vaccines targeting especially toward intracellular pathogens (mycobacteria, virus, parasites) for which the old technologies are still ineffective nowadays.

Reports of adjuvants that have been tested with promising results are (Tables 1 and 2), e.g. the liposomal adjuvants systems, such as **CAF01**, a report by van Dissel et al.[92] as adjuvant of a vaccine formulation against *M. tuberculosis*, Ag85B-ESAT-6 (H1) represents a first-in-man trial, that induced a Th1 response and antigen-specific T-cell responses of long-lasting as immunological memory after 150 weeks. In another study, the adjuvant IC31(®), plus Ag85B-ESAT6 boost individuals previously vaccinated with BCG and those latently infected with TB elicited strong antigen-specific T cell responses against Ag85B-ESAT-6 and both the Ag85B and ESAT-6 components that could be augmented by the second vaccination. The strong responses persisted through

Table 2. Adjuvants in clinical phase studies of human infectious disease vaccines.

Adjuvant	Type	Phase of clinical study	Applications
Mineral salts			
-Alum salts		Pilot, Phase I, I/II, II, II/III, III, IV/	Gram-positive (*e.g.Staph, Clostridium*) Gram-Negative (e.g. *C. jejuni, H. pylori*
-Freund's Incomplete	Prophylatic/therapeutic	Phase I, I/II, II, III	Virus (hepatitis, HIV, West nile) Parasites (Malaria, Leishmaniasis)
		Phase I, I/II, II, III	Virus (influenza)
		Pilot, Phase I, I/II, II, II/III, III	Parasites (Malaria
TLR Agonists			
Monophosphoryl lipid A (MPL)	Prophylatic/therapeutic	Phase I, I/II, II, III/ Phase I, I/II, II, III	Virus (hepatitis, HIV, herpes, Norovirus) Parasites (Leishmaniasis)
Flagellin	Prophylatic	Phase I, I/II, II, III	Virus (e.g. influenza, dengue)
Poly (I:C)	Prophylatic/therapeutic	Phase I/II, Pilot, I, I/II, II	Virus (influenza)
Imiquimod	Prophylatic/therapeutic	Phase II, II/III, III/ Phase I, I/II, II, III, IV	Virus (influenza, varicela, hepatitis B)
Resiquimod	Prophylatic/therapeutic		Virus (influenza, hepatitis B)
Muramyl dipeptide	Prophylatic	Phase I	Virus (HIV)
CpGODN IC3I	Prophylatic/therapeutic	Phase I, I/II	Bacteria
		Phase I, I/II, II	Virus (HIV, hepatitis B) Parasites (Malaria)
Emulsions			
MF59	Prophylatic/therapeutic	Phase I, I/II, II, II/III, III, IV/I	Virus (respiratory, HIV, influenza, citomegalovirus)
QS21			Virus (HIV, gp120) Parasites (Malaria)
Detox			Parasites (Malaria) (R32NS18)
Particle systems			
Virosomes	Prophylatic	Phase I, I/II, II, III, IV	Fungi (Candidiasis) Parasites (Malaria Virus (Hepatitis B, C, influenza)
Virus-like particles	Prophylatic/therapeutic	Phase I, I/II, II, III, IV Phase I, I/II, II	Parasites (Malaria Virus (Papillomavirus,Norovirus, Enterovirus 71)
Adjuvant systems			
ASO4 IC3I	Prophylatic/therapeutic	Phase I, II, III, IV Phase II/III	Virus (hepatitis B)
ASO3	Prophylatic	Phase I, I/II, II Phase III, IV	Virus (influenza, dengue)
AS02A		Phase III, I	Parasites (Malaria)
AS01B		Phase I	Mycobacteria (Tuberculosis)

Luchner et al., 2021; De Souza et al., 2016; O'Hagan and Valliant et al., 2013.

32 weeks of follow-up, implied the induction of long-lasting immunological memory (Table 2).[92,93] Molecular adjuvants such as type I IFNs, bacterial toxins[10,14,31,70–74,78] (Table 3) can reach the inductive sites is because they interact with receptors like molecules on the antigen-presenting cells (APC) and thus, triggering directly signalization to the nucleus for the production of the inflammatory response which links thus, with the adaptive immune response (humoral and cellular). The host response is fast, and it did not allow the expression of the bacterial resistance genes on the contrary it leads to an effect that can last in the activation of the surveillance mechanism of defense innate response. Work from us in the field of candidates adjuvants vaccines of BCG against *Mycobacterium tuberculosis* (*MTb*) have shown that immunodominant antigens such as HBHA (Table 3) plus *M. b*ovis BCG vaccine, represent a synergistic system, for one side a mycobacterial antigen that enhanced BCG vaccine immunity against *M. tuberculosis*, acted through a bacteriostatic effect, that is, limiting the growth and at the same time exert immunomodulation of the Th1 type cellular immune response.[94–96] Another *in vivo* study on this same concept is the systemic adjuvant effect of type I IFNs in a murine model of leprosy[31]or tuberculosis[32] (Table 3).

On the other hand, adjuvants in SARS-Cov2 vaccine formulation. There are more than 100 COVID-19 vaccines under development and using a different platform as inactivated virus, recombinant proteins, viral vectors, ADN, RNA, and others and, some of them already authorized to use in humans (Table 3). Two vaccines based on inactivated SARS-Cov2, have more advanced results. Thus, the clinical trials ChiCTR2000031809/ChiCTR2000032459 performed by the Wuhan Institute of Biological Products/Sinopharm tested inactivated SARS-Cov2 adjuvanted with alum. In phase I/II clinical trial, this vaccine-induced high titers of antibodies in immunized individuals. The vaccine was safe, well-tolerated and, neutralizing antibody response was higher after two doses with 4ug than single doses with 8ug. It is approved in China for emergency use.[97,98] The SINOVAC/CORONAVAC vaccine in phase I/II trial used two different doses 3ug or 6 ug antigen administered intramuscularly in a regimen including prime-boost at 2 and 4 weeks. Aluminum hydroxide was used as an adjuvant demonstrating safety and immunogenicity without adverse events. Overall, more than 90% of immunized individuals

Table 3. From old to new adjuvants compounds in preclinical testing (animal studies) for human vaccines.

Adjuvant	Type	Experimental/ Preclinical	Application	Reference
Triterpenes (QS-21)			*Staphylococcus* *Streptococcus* *Pseudomonas* *H. Pylori*	Lacalli-Dubois et al.,[22] Sugai et al.[12] Longhi et al.[11] Lee et al.[9]
Poly (I:C) Toxins Chemokines	prophylatic	mouse/*in vivo*	*HIV* *Cytomegalovirus* *Influenza* *ARS* *Malaria* *Leishmaniasis*	Mohan et al.[3]
Type I IFNs	prophylatic	mouse/*in vivo* A549, THP-1 /*in vitro*	Influenza *M. tuberculosis* *M. lepraemurium*	Bracci et al.[26] Guerrero GG et al.[31] Rivas-Santiago and Guerrero[32])
nHBHA rHBHA	prophylatic	mouse/*in vivo*	*M. tuberculosis*	Locht et al.[92] Guerrero et al.[93] Guerrero and Locht[94]
Bt Cry proteins	prophylati	mouse/*in vivo*	*Naegleria fowleri* *Malaria* *M. bovis* BCG	Rojas-Hernandez et al.[78] Legorreta et al.[81] Ibarra-Moreno et al.[79] Gonzalez-Gonzalez et al.[80] Favela-Hernandez et al.[14])
mRNA platform Vaccine intrinsic adjuvanticity			SARS-Cov2	Topol[95] Polack et al.[96]

*CpG-ODN, umethylated oligonucleotides; Poly(I:C)(Polyinosinic:polycytidylic acid), nHBHA, native heparin binding haemaglutinin, rHBHA, *E.coli* recombinant heparin binding hemagglutinin, type I IFNs, Interferons alpha/beta, *Bt* Cry proteins, *Bacillus thuringiensis* Cry proteins

generated binding antibodies (NCT04352608 accessed 10 April 2021)[99].

The innovative application of nanomaterials as vaccine adjuvants have been increasingly investigated for immune protection and immunotherapy for infectious diseases. Thus, Novamax (USA) is testing Matrix-M1 which is composed of nanoparticles of saponin from the *Quillaja saponaria*, cholesterol and, phospholipids in perfusion-stabilized combined to full-length spike protein SARS-Cov2 in phase I /II. The results demonstrated that immunized individuals elicited a higher titer of neutralizing antibodies than the convalescents and the T cell response elicited was Th1 polarized. Phase II studies are still running (NCT04368988 accessed 10 April 2021).[100] Several ongoing clinical trials (phase I) are using a variety of adjuvants previously tested in other infectious diseases. AS03 (squalene based) and CpG 1018 (based on TLR9 agonist)/Alum combined with an innovate antigen recombinant SARS Cov2-trimeric spike protein produced in a mammalian cell culture-based expression system is applied via intramuscular route at 1 and 22 d (Clover Biopharmaceuticals Inc./ GSK/Dynavax, NCT04405908 accessed April 10, 2021). Similarly, KBP-Covid 19 (Kentucky Bioprocessing, Inc) plus CpG adjuvant is evaluated in Phase I in healthy seronegative individuals (NCT04473690 accessed 10 April 2021).

On the other hand, the vaccine developed by GSK/Dynavax is testing MF59 oil emulsion plus SARS CoV2 Spike protein stabilized with a molecular clamp (ACTRN1262000067 accessed 10 April 2021). Previous studies in mice demonstrated high neutralizing antibody titers and polyfunctional T cell CD4 and CD8 T cell response. Vaccine, use a novel adjuvant produced from microparticles of delta inulin (β-D-(2->1)-poly-fructo-furanosyl-D-glucose) named Advax-SM combined with a monovalent recombinant COVID-19 spike protein has been tested in recruiting individuals (NCT04453852 accessed 10 April 2021). Moreover, an mRNA vaccine platform with intrinsic adjuvanticity effect against prostate cancer and/or in SARS-Cov2, a TLR agonist of TLR-3, TLR-7, and TLR-8 have been also reported.[101–103]

In COVID-19, still is challenging to develop a vaccine to prevent infection. All authorized vaccines for emergency uses only to prevent severe disease progression. It is essential to identify the suitable adjuvant in the SARS-Cov2 vaccine development to induce the appropriate immune response able to protect against the disease acquisition

5. Remarks and perspectives

The interaction host-pathogen is a potentially rich source of candidates and targets for the development of innovative, experimental, and bioinformatic technologies to provide a more rapid and accurate tool for the development of the most safety profile of adjuvants compounds an important component of modern vaccines. The validations of the aforementioned adjuvants enable the capacity to initiate, connect and boost host immune response to infectious diseases. These compounds can help to induce effective, prolonged, and specific immune responses (humoral and cellular) especially to a bystander and/or poor antigens, especially in elderly, vulnerable people that are a poor response to unadjuvanted vaccines. Optimization, and safety profiles, benefit:risk ratio, epidemiological survey and studies, and deep evaluation of the preclinical studies that include all experimental settings in the different animal models, should be followed and taken into account for further exploration and investigation. Nowadays, mineral salts (Freund's adjuvant, alum salts), continue to be used in some formulations; however, it is more evidence that there are more alternatives such as that molecular adjuvants, type I IFNs, bacterial toxins, mRNA platform with intrinsic adjuvanticity that, in combination with nanotechnology, represent a friendly and promising strategy for clinical use (future scenery).

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